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Relationship of foetal number and parity in Barbari goats to plasma profile of caprine pregnancy-associated glycoprotein (caPAG) during gestation and the early postpartum period

S.P. Singh[a,*], N. Ramachandran[a], N. Sharma[a], A.K. Goel[a], N.M. de Sousa[b], J.F. Beckers[b], D.K. Swain[c], M.K. Singh[d], S.D. Kharche[a]

[a] Animal Physiology and Reproduction Division, ICAR-Central Institute for Research on Goats, Makhdoom, Farah, Mathura, Uttar Pradesh, India
[b] Laboratory of Animal Endocrinology and Reproduction, Faculty of Veterinary Medicine, University of Liege, 4000, Belgium
[c] Department of Veterinary Physiology, College of Veterinary Science & Animal Husbandry, UP Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, Uttar Pradesh, India
[d] Animal Genetics and Breeding Division, ICAR-Central Institute for Research on Goats, Makhdoom, Farah, Mathura, Uttar Pradesh, India

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ABSTRACT

This study was conducted to characterise pregnancy-associated glycoprotein (caPAG) in peripheral plasma during gestation and postpartum periods of nulliparous and multiparous does with one or two foetuses using a caPAG specific two-step sandwich ELISA system. Earliest time-points for detection of pregnancy and foetal number with appropriate cut-off values were identified. Plasma samples from 15 pregnant (multiparous: $n = 8$; nulliparous: $n = 7$; during pregnancy and postpartum period) and six non-pregnant (during oestrous cycle) goats were collected and analysed. Mean caPAG concentration was greater than the threshold for pregnancy detection (S-N = 0.40) on d22, peaked on d45 and remained unchanged until parturition. From d45 until parturition, caPAG concentration in multiparous does with two foetuses was 1.4 to 1.8 fold greater ($P < 0.001$) than those with one foetus. For the ELISA, 0.83 (S-N) was the most appropriate cut-off to differentiate does with two from those with a single foetus with an overall sensitivity and accuracy of 88.9% and 84.7%, respectively. Circulating caPAG concentration in multiparous goats was greater ($P < 0.05$) compared with nulliparous goats during the early pregnancy and postpartum periods. After parturition, caPAG concentrations markedly decreased and were basal within 14 days postpartum. In conclusion, using the caPAG specific ELISA, results indicated there were unique gestational and postpartum profiles for caPAG concentrations that are affected by number of foetuses and parity of the doe. The marked decrease in concentration of caPAG following parturition indicates there would not be compromising of the detection of subsequent pregnancies in goats using this technique.

1. Introduction

Effective strategies to improve reproductive efficiency of goats involve early identification of pregnancy status and re-breeding of non-pregnant does to reduce the number of days animals are not pregnant. In farm animals, including goats, the placenta functions as

* Corresponding author at: Animal Physiology and Reproduction Division, ICAR-Central Institute for Research on Goats, Makhdoom, Farah, 281 122, Mathura, Uttar Pradesh, India.
E-mail address: shiva.singh@icar.gov.in (S.P. Singh).

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an endocrine and paracrine organ by secreting a wide range of chemical messengers comprising proteins and growth factors into the maternal circulation (Hoffmann and Schuler, 2002).

Pregnancy-associated glycoproteins (PAGs), a placental-derived family of inactive aspartic proteinases, are abundantly produced and released by the trophoblastic placental layer during pregnancy in ruminants (Wooding et al., 2005; de Sousa et al., 2006; Haugejorden et al., 2006). Even though PAG concentrations can be detected at early gestation in cattle (Szenci et al., 1998), sheep (Roberts et al., 2017) and goats (Tandiya et al., 2013), accuracy of pregnancy diagnosis before d 30 after conception can be compromised by individual differences in PAG secretion by placental cells (Chavatte-Palmer et al., 2006), as well as when there is embryonic mortality during this sensitive period of gestation in goats when there is maternal recognition of pregnancy (Samir et al., 2016). Furthermore, the persistence of some members of the PAG group during the postpartum period may also limit the use of existing methods (de Sousa et al., 2003).

It is important to characterise the PAG profile in farm animals using specific assay systems. This is mainly because the placental functions are related to the maternal parameters (including parity and litter size) and breed (Dwyer et al., 2005). Furthermore, variation in the results when there is use of PAG assays are sometimes attributed to the specificity of antibodies used in assay systems (Gonzalez et al., 2000; Karen et al., 2015; Gatea et al., 2018). The use of specific antibodies in the assay system, therefore, could permit accurate detection of early pregnancy and identification of the number of foetuses in goats.

The determination of number of foetuses *in utero* is desirable for more precise management of pregnant does and for achieving more profitable production of products derived from goats. Results of some previous studies, however, indicated certain differences in mean PAG concentrations in does with one or two foetuses (de Sousa et al., 1999; Batalha et al., 2001b). Due to large individual variation, PAG concentrations during pregnancy in does with a single foetus, however, there cannot be differentiation from does with multiple foetuses (Barbato et al., 2009). Hence, determination of caprine PAG (caPAG) concentrations with an appropriate cut-off value is required to distinguish between does with one or two foetuses. Information related to the effect of number of foetuses and parity on the plasma profile of caprine PAG (caPAG) throughout gestation and the postpartum period in tropical goats is not available. The objectives of the present study, therefore, were to (1) characterise the temporal profile of caPAG concentrations in nulliparous and multiparous does with one or two foetuses throughout pregnancy and the early postpartum period using a caPAG specific sandwich ELISA system and (2) determine the earliest time points with appropriate cut-off values for pregnancy detection and determination of does with one or two foetuses.

2. Materials and methods

2.1. Experimental animals and animal management

Altogether, 21 female Barbari goats were included in the study with either a confirmed pregnancy [$n = 15$ (nulliparous: $n = 7$; weighing 25.9 ± 2.2 kg and multiparous (3–4 parity): $n = 8$; weighing 28.6 ± 1.9 kg)] or confirmed to not be pregnant ($n = 6$; weighing 26.8 ± 1.6 kg). Transrectal ultrasonography (USG) was used for the determination of pregnant and non-pregnant animals at 37 days post-mating. The animals were maintained in a group with a semi-intensive system of management being imposed for which there was uniform nutritional conditions with free access to water throughout the experiment. Goats were bred 10–12 h after onset of natural oestrus by bucks of the same breed. After parturition, all does in the experiment nursed their kids until the end of the study. All the experimental procedures were conducted using veterinary practices that were approved by the Animal Ethics Committee of the Institute.

2.2. Blood sampling and storage

Blood samples from the animals were collected on an alternate day from d 16 after breeding until the end of the first month. During the second (> d 30 to d 60) and subsequent months (d 75 until parturition) blood samples were collected once a week and at 15 day intervals, respectively. To study the postpartum concentrations of caPAG, blood samples were collected on an alternate day for the first week and subsequently at weekly intervals until 28 days after parturition. To estimate the accuracy of caPAG assay, blood samples ($n = 88$) from six non-pregnant Barbari goats of the same flock with similar genetic merit for productivity were collected during different days of the oestrous cycle. For each sampling, a volume of 5 mL of blood was collected via jugular venepuncture into 10 mL plasma dipotassium (K2)-EDTA evacuated tubes (Vacutainer, BD, Franklin Lake, NJ) and immediately placed in the icebox. After centrifugation at 2000 × *g*, 10 min at 4 °C, plasma was harvested and stored at −20 °C until being assayed.

2.3. Ultrasonographic (USG) assessment

Transrectal USG was performed in all the animals on d 37 post-mating with use of an ultrasonic device (Just Vision 200-Model SSA- 320A, Diagnostic Ultrasound System, Toshiba, Japan) equipped with a real-time convex array transrectal transducer (PVF-738 F) of variable frequency (5–7 MHz), as previously described (Goel et al., 2009). There were subsequent evaluations of the non-pregnant uterus to eliminate the chances of a false negative diagnosis. Blood sampling from pregnant animals, as observed by USG examination, was continued until the end of the experiment.

Table 1
Characteristics of antigen preparations used to generate the two caprine pregnancy-associated glycoprotein specific antisera used for the ELISA.

AS	PAG	N-terminal micro-sequence	Accession No.	pI
707	$caPAG_{55kDa}$	ISSPVSXLTIHPLRNIMDMLYVGXITI	P80935	5.3, 5.1, 4.9
	$caPAG_{62kDa}$	RDSXVTIVPLRNMRDIVYVGXITIGTP	P80933	6.2, 5.9, 5.6
708	$caPAG_{55kDa}$	ISSPVSXLTIHPLRNIMDMLYVGXITI	P80935	5.3, 5.1, 4.9
	$caPAG_{59kDa}$	RGSXLTTLPLRNIMDMLHMGXITIGTP	P80934	5.1, 4.8

AS = antiserum; caPAG = caprine pregnancy-associated glycoprotein; pI = Isoelectric Point.

2.4. Generation of antibodies

Two mature New Zealand white rabbits (AS#707 and AS#708) were immunised with distinct purified PAG preparations by intradermal route (Vaitukaitis et al., 1971). For the first immunisation, 500 µg of proteins were dissolved in 1.0 mL phosphate buffer 0.05 M (pH 7.5) and emulsified with Freund complete adjuvant (Difco Labs, Detroit, MI, USA). Booster doses (200 µg) were injected at 3 and 4 week intervals (Freund incomplete adjuvant). Blood was collected from the marginal ear vein starting 1 month after the second injection and sampling continued at monthly intervals until 10 months. Rabbit blood samples were allowed to clot overnight at room temperature. Samples were centrifuged at 1500 × *g* for 20 min, and the sera were stored at −20 °C until used.

2.5. Development of a caPAG-ELISA

The two-step immunometric sandwich ELISA was established for the detection of caPAG using a paired combination of caPAG specific rabbit anti-PAG polyclonal antisera [i.e., PAG#707 (anti-$caPAG_{55+62\ kDa}$)] and PAG#708 (anti-$caPAG_{55+59\ kDa}$). The details about the antisera are presented in Table 1. The antiserum PAG#707 was used for coating of microtiter plates and the biotin labelled purified IgG (PAG#708) was used as a conjugated detection antibody. For biotin labelling, the lyophilised antisera (PAG#708) was first reconstituted in 50 mM sodium bicarbonate buffer (pH 8.2) and the immunoglobulins were purified using Protein-A based IgG purification kit (Himedia, MBPP001SP). After the determination of protein concentration by BCA method (Merck Millipore, 71285-3), the purified immunoglobulins were labelled with biotin (Sigma, Mix-n-Stain™ Biotin antibody labelling kit; MXBIOS100), following the manufacturer instructions.

For the assay, high binding 96 well microtiter plates (EIA plate 9018; Corning Costar, Cambridge, MA, USA) were coated with rabbit anti-caPAG polyclonal antibody (PAG#707) in coating buffer and allowed to incubate overnight at 4 °C in the moist condition. After blocking of free binding sites with 2.5% casein in 0.05 M NaCl, pH 7.3 (200 µL/well) at 37 °C for 1 h (Digital incubator T-701, Shivaki), the plates were then washed five times with wash solution (250 µL/well for each wash; PBS containing 0.05% Tween 20; Plate washer Hydroflex, Tecan). After washings, the coated microtiter plates were used immediately for PAG assay.

First, 50 µL of assay buffer [0.12 M NaCl, 0.02 M Na_2HPO_4, 0.01 M di Sodium EDTA, 0.005% chlorhexidine digluconate (20%), 0.002% phenol red, 0.1% Gelatin, 0.05% Tween 20 and 0.02% ProClin 150] were pipetted into each well. An equal volume of plasma samples (in duplicates) and assay negative control (pool female kid plasma; in six different predefined wells) were subsequently added and incubated for 90 min at 37 °C. After incubation, plates were washed three times with 250 µL wash solution. The conjugate solution (100 µL/well; biotin labelled anti-PAG antibody, AS#708) was added and incubated for 1 h at 37 °C. After four washings, 100 µL of detector solution (assay buffer with Avidin–Peroxidase, Sigma, A3151) was pipetted and incubated for 1 h at 37 °C. After five washings, the substrate solution containing 0.05 M citric acid, 0.055 M Na_2HPO_4, 0.05 M H_2O_2, 0.02% ProClin 150, and 0.025% of TMB (12.5 mg 3,3′5,5′-Tetramethylbenzidine/mL DMSO) was added and incubated for 30 min at RT in a darkened area. The reaction was stopped with 50 µL of 1 M oxalic acid and optical density (OD) was determined at 450 nm with a microtiter plate reader (Tecan Sun Rise with Magellan 4.0 Software, Austria).

2.6. Results interpretation

Results interpretations were performed as described previously (Commun et al., 2016). Briefly, OD values of each sample in duplicate and six ODs of negative control were determined at 450 nm and a reference wavelength of 630 (620–650) nm. Corrected OD values for all samples and controls were measured as the OD value at 450 nm minus the OD value at the reference wavelength (650 nm). A 'signal minus noise (S-N)' value was then determined for each sample by subtracting the mean of the corrected negative control OD from the mean corrected OD of the sample.

2.7. Analytical validation

Analytical validation of the ELISA was achieved by determining reproducibility [coefficient of variation (CV) within a plate (intra-assay CV or repeatability) and between plates (inter-assay CV or precision)], recovery and dilutional linearity. Furthermore, different variables for the assessment of predictive values of the assay were estimated.

2.7.1. Reproducibility

Reproducibility was determined by repeated analysis of different plasma samples measured in assays and expressed as per cent intra- or inter-assay CV [%CV = (Standard deviation/Mean) × 100], respectively.

For determination of intra-assay CV, four plasma samples with different caPAG concentrations were tested at forty positions of two assay plates. Similarly, for the determination of inter-assay CV, twelve plasma samples were determined (in duplicate) on different days. The acceptable concentrations of intra-assay CV and inter-assay CV for ELISA were considered as ≤ 10% and ≤ 20%, respectively (Ghosh et al., 2015).

2.7.2. Percent recovery of the assay

The recovery was determined by the repeated analysis of three plasma samples with predetermined S-N values (high, mid and low). For this determination, 12 different combinations of these samples were prepared by pooling two samples equally (50:50) at a time and measured in the assay. The percentage recovery was determined by comparing the S-N values of the assay with the predetermined values and calculations were made using the formula [(observed value - expected value)/expected value] × 100.

2.7.3. Dilutional linearity

Four plasma samples with varying caPAG concentrations were used for the assessment of assay linearity. All samples were used undiluted as well as diluted in different concentrations (i.e., 1:2, 1:3, 1:4 and 1:5) with assay buffer and subjected to the ELISA tests. The dilutional linearity was calculated from the CV of observed S-N values.

2.7.4. Estimation of predictive values

The predictive values of the test were determined using procedures that have been previously described (Ricci et al., 2015). Briefly, the positive predictive value (PPV) [PPV = TP/(TP + FP)], negative predictive value (NPV) [NPV = TN/(TN + FN)] and accuracy [(TP + TN)/(TP + TN + FP + FN)] were calculated and expressed as a percentage. The TP, FP, TN and FN represent true positive, false positive, true negative and false negative results, respectively.

2.8. Statistical analyses

For statistical analyses and presentation of results, the entire experiment was divided into early gestation (d 16 to d 37 of pregnancy), mid and late gestation (d 45 of pregnancy to parturition) and days after parturition (d 1 to d 28 postpartum). The 'Linear Mixed Model' procedure was used for parity and number of foetuses as a dependent variable. 'Treatment' (parity of doe or number of foetuses) and 'Time' (day post-breeding or postpartum) as the fixed factors, sampling time as a repeated effect, does as a random factor and the respective interactions were included in the model. The diagonal covariance structure followed by utilising the Bonferroni correction was used to estimate level of significance of treatment, time and their interaction for each dependent variable. For comparisons of caPAG concentration among the groups at an individual time point, an independent sample *t*-test or corresponding non-parametric test (Mann–Whitney U test) was performed. Differences were considered significant at the probability value (P) < 0.05. All data are presented as the mean ± standard error of the mean.

The SPSS-20 software was used to create Receiver Operating Characteristic (ROC) curves where pregnancy and two foetuses were designated as the true positive. For the analysis of results, a cut-off value of S-N was fixed as < 0.4 as non-pregnant and ≥ 0.4 as pregnant does (for entire gestation), and < 0.85 as does with a single foetus and ≥ 0.85 as does with two foetuses. The association between plasma caPAG concentration and number of foetuses was determined by using binary logistic regression models. The values of 'goodness of fit' of logistic regression models were tested using the Hosmer–Lemeshow method.

3. Results

Pregnancy in the goats was using USG, caPAG concentration quantifications utilising the ELISA developed for this study and ultimately with does delivering kids at the time of parturition. Of 21 does, seven had a single foetus, eight had two foetuses and six were non-pregnant. The overall mean gestation period of does was 144.1 ± 1.6 days (138–150 days). The mean gestation lengths for does with one or two foetuses were 145.5 ± 1.27 (139–150 d) and 142.6 ± 3.1 (138–148 d), respectively.

3.1. Validation of PAG ELISA

Intra- and inter-assay CVs were 4.06% and 9.08%, respectively. With recovery of PAG assessments, these ranged from 79.1% to 121.1% (Mean: 99.2 ± 6.6%). For estimation of dilutional linearity, four plasma samples with different PGA concentrations (1.28, 1.34, 1.37 and 2.12) were used. The concentration of PAG was estimated in plasma samples and in tubes where there were dilutions in the assay buffer (1:2, 1:3, 1:4 and 1:5). The CV of plasma samples and the respective dilutions ranged from 9.8% to 17.8% (mean CV = 13.1 ± 1.9%). A representative serial dilution curve of a plasma sample is depicted in Fig. 1.

3.2. Evaluation of the ROC curve and calculation of AUC

One of the aims when conducting the present study was to determine S-N cut-off values for early pregnancy detection and identification of the number of foetuses. For this, ROC curve an analysis was conducted for different cut-off values to obtain the most

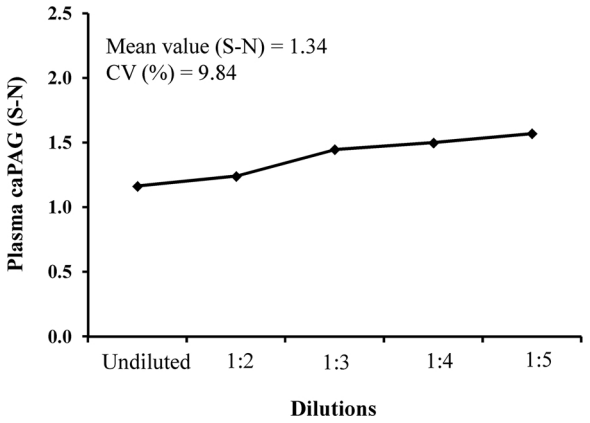

Fig. 1. Representative serial dilution curve of plasma samples with linearity at different dilutions of a plasma sample (undiluted, 1:2, 1:3, 1:4 and 1:5) with the assay buffer.

precise combination of sensitivity and specificity (Fig. 2). The analysis resulted in an optimal cut-off S-N value of 0.40 for the assay with AUC of 0.703, 0.864, 0.985 and 0.891 for different pregnancy periods (Table 2). The data for PPV, NPP and accuracy of plasma caPAG ELISA outcomes during pregnancy are provided in Table 3. Similarly, based on the most appropriate values of sensitivity (88.9%), specificity (70.4%), PPV (84.0%), NPV (86.4%) and accuracy (84.7%), the cut-off value for caPAG concentrations to discriminate multiparous does with one or two foetuses from ≥ d 45 of gestation was 0.830 (Table 4). The area under the ROC curve (0.897; $P < 0.001$) indicates the excellent foetal number discrimination capacity of the assay. The logistic regression test indicated an excellent association ($P < 0.001$) between plasma caPAG concentration and the number of foetuses during mid- and late-gestation (i.e., ≥ d 45 of pregnancy in multiparous goats).

3.3. Plasma PAG profiles

To determine the temporal profile of circulating caPAG during different stages of pregnancy and after parturition in does pooled data from does with one or two foetuses were analysed and data are depicted in Fig. 3. The caPAG profile was characterised by a rapid increase during early gestation (from d 16 to d 37) and attainment of the greatest concentration on d 45 of pregnancy. The concentration of caPAG remained at this relatively greater value until the last observation before the day of parturition (i.e., d 135; Fig. 3). After parturition, there was a marked decrease in caPAG concentration with basal concentrations occurring within 14 days

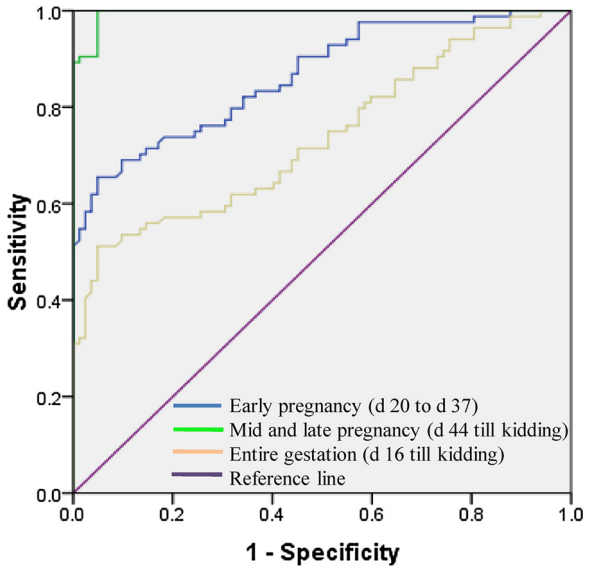

Fig. 2. Receiver Operating Characteristic (ROC) curve of circulating caprine pregnancy-associated glycoprotein (caPAG) quantified using an ELISA in pregnant Barbari does during gestation; A plasma caPAG concentration (S-N value) of 0.4, resulted in 70.2% (blue line), 97.5% (green line), and 80.2% (pink line) sensitivity during early- (d 20 to d 37), mid- and late-gestation (d 45 until parturition) and entire gestational period (d 16 until parturition) with an area under the curve of 0.864, 0.971 and 0.859 ($P < 0.001$), respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Sensitivity and specificity of caPAG ELISA for pregnancy detection based on the circulating PAG concentration assessed on different days of gestation at various threshold values.

Days of pregnancy*	PAG-threshold values	Se (%)	Sp (%)	AUC and *P* value	95% CI	
					Lower bound	Upper bound
16-20 days	0.250	66.0	55.3	0.703 ($P < 0.001$)	0.603	0.803
	0.300	61.7	62.8			
	0.350	57.4	71.3			
	0.400	53.2	86.3			
22-37 days	0.250	90.5	52.4	0.864 ($P < 0.001$)	0.811	0.918
	0.300	83.3	59.8			
	0.350	77.4	69.5			
	0.400	70.2	86.6			
≥ 45 days	0.250	99.2	52.4	0.985 ($P < 0.001$)	0.971	0.999
	0.300	99.2	59.8			
	0.350	97.5	69.5			
	0.400	97.5	86.6			
Overall	0.250	90.1	52.8	0.891 ($P < 0.001$)	0.859	0.923
	0.300	86.6	60.2			
	0.350	83.0	69.4			
	0.400	80.2	85.2			

Se = Sensitivity; Sp = Specificity; AUC = area under curve; CI = confidence interval.

Table 3
Positive predictive value (PPV)[1], negative predictive value (NPV)[2], and accuracy[3] of caprine pregnancy-associated glycoprotein (caPAG) ELISA tests for determination of pregnancy status at different stages of gestation in Barbari goats (total number of samples screened = 425).

Pregnancy stage	PPV [% (no./no.)]	NPV [% (no./no.)]	Accuracy [% (no./no.)]
d 16-20	0.59 (26/44)	0.79 (70/89)	0.72 (96/133)
d 22-until kidding	0.91 (179/197)	0.74 (70/95)	0.85 (249/292)
Overall (d 16 to kidding)	0.85(205/241)	0.76 (140/184)	0.81 (345/425)

[1] Proportion of plasma samples of pregnant Barbari goats with caPAG greater than the cut-off value (S-N = 0.40).
[2] Proportion of plasma samples of non-pregnant Barbari goats with caPAG less than the cut-off value (S-N = 0.40).
[3] Proportion of number of samples that was correctly classified using the assay based on the results of total number of plasma samples screened.

postpartum.

Mixed model analysis along with pairwise comparison indicated there was a difference ($P < 0.001$) between ewes with one or two foetuses for multiparous does during mid- and late-gestation (Fig. 4). The effect of number of foetuses on plasma caPAG was first observed on d 45 of pregnancy, and this difference continued to exist until the end of gestation. The plasma caPAG concentrations was 1.4–1.8 fold greater (during mid and late gestation) in does with one as compared with two foetuses. Whereas, the difference in the caPAG concentration among the groups was not observed during early gestation (d 16 to d 37; $P = 0.702$) and after parturition (d 1 to d 28 postpartum; $P = 0.751$; Fig. 4). Overall, in the present study, plasma caPAG concentrations in pregnant multiparous does were affected by both number of foetuses (one or two; $P = 0.004$) and the stage of gestation ($P < 0.017$).

When data were analysed to evaluate the effect of parity on caPAG concentration during pregnancy and the postpartum period in does with one foetus, plasma caPAG concentration was affected ($P < 0.001$) by parity (Fig. 5). The mean caPAG concentrations increased progressively in both nulliparous and multiparous does and were at a maximum concentration at about d 45 of pregnancy. The concentration of caPAG, however, was less ($P < 0.001$) in nulliparous compared with multiparous does during early pregnancy and after parturition, but not during mid- and late-gestation periods (Fig. 5). The plasma caPAG concentration in nulliparous does was 0.9–2.9 fold (during pregnancy) and 1.3–2.6 fold (after parturition) less compared with multiparous does. Similarly, the rate of decrease in caPAG concentrations during the first week of the postpartum period was 1.6 fold greater in nulliparous than multiparous does. Due to the more rapid decrease of caPAG concentrations, during the postpartum period in nulliparous does there were basal values of caPAG much earlier (within 7 days) than in the multiparous (around 21 days postpartum) does.

4. Discussion

New methods to identify pregnancy status and foetal number may be important in further optimisation of management practices to improve profitability of goat farms. As an alternative to USG, therefore, laboratory methods based on detection of specific antigens, such as PAGs, are increasingly used for farm animals.

The present study was designed to detect early pregnancy and the number of foetuses in does with the use of a new matched pair of two caPAG specific rabbit antisera in a two-step sandwich ELISA system for detection of PAG in peripheral blood of pregnant does having different numbers of parities. By using the ELISA that was developed in the present study, there was detection of a gradual

Table 4
Sensitivity, specificity, positive predictive value (PPV)[1], negative predictive value (NPV)[2], and accuracy[3] of caprine pregnancy-associated glycoprotein (caPAG) ELISA tests for determination of number of foetuses based on the various threshold values of circulating PAG concentration (S-N) assessed during mid- and late-gestation in Barbari goats (total number of samples screened = 72).

Days of pregnancy	PAG-threshold values	Se (%)	Sp (%)	PPV [% (no./no.)]	NPV [% (no./no.)]	Accuracy [% (no./no.)]	AUC and *P* value	95 % CI	
								Lower bound	Upper bound
≥ 45 days till kidding	0.750	100	55.6	78.9 (45 / 57)	100 (15 /15)	83.3 (60/72)	0.897 ($P < 0.001$)	0.826	0.968
	0.800	95.6	66.7	82.7 (43/52)	90.0 (18/20)	84.7 (61/72)			
	0.830	88.9	70.4	84.0 (40/ 50)	86.4 (19 / 22)	84.7 (61 / 72)			
	0.900	73.3	81.5	86.8 (33 / 38)	64.7 (22 / 34)	76.4 (55 / 72)			

Se = Sensitivity; Sp = Specificity; AUC = area under curve; CI = confidence interval.
[1] Proportion of plasma samples of single foetus bearing Barbari goats with caPAG less than the threshold value.
[2] Proportion of plasma samples of twin foetuses bearing Barbari goats with caPAG greater than the threshold value.
[3] Proportion of number of samples that was correctly classified using the assay based on the results of total number of plasma samples analysed.

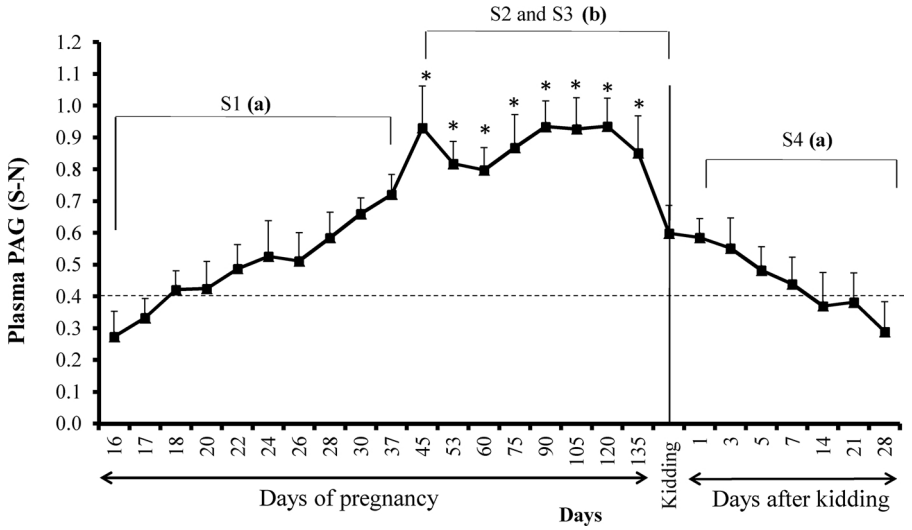

Fig. 3. Profile of circulating caprine pregnancy-associated glycoprotein (caPAG) (mean signal-noise; S-N ± SEM) quantified using an ELISA in pregnant Barbari goats during and after gestation; S1 = pregnancy stage 1 (early pregnancy: to d 37); S2 and S3 = pregnancy stage 2 (mid-pregnancy: from d 45 to d 90) and 3 (late-pregnancy: from d 105 to day of parturition), respectively; S4 = postpartum period (d 1 to d 28 of lactation); Asterisk represents differences ($P < 0.05$) between time points and different letters (a, b) indicate differences ($P < 0.01$) among different stages (S1, S2, S3, and S4) during and after pregnancy.

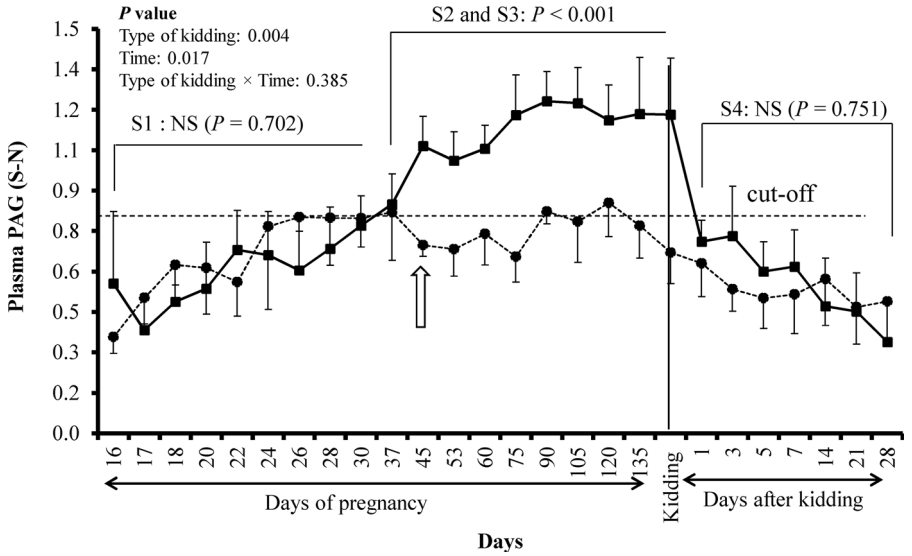

Fig. 4. Profiles of circulating caprine pregnancy-associated glycoprotein (caPAG) (mean signal-noise; S−N ± SEM) quantified using an ELISA developed when conducting the present study when does had one (dotted line and closed circle; $n = 3$) or two (solid line and closed square; $n = 5$) foetuses in multiparous Barbari goats during and after gestation; S1 = pregnancy stage 1 (early pregnancy: up to d 37); S2 and S3 = pregnancy stage 2 (mid pregnancy: from d 45 to d 90) and pregnancy stage 3 (late pregnancy: from d 105 to day of kidding), respectively; S4 = days after kidding (d 1 to d 28 postpartum); NS represents no significant difference between the groups during early pregnancy and after parturition; Type of kidding represents does with one or two foetuses; The arrow and cut-off line indicate the earliest time point and the S−N value (0.83) to determine the number of foetuses, respectively.

increase in caPAG concentrations during early pregnancy. Overall, the caPAG concentration in circulation is greater than the cut-off value on d 22 and subsequently when there was the greatest concentrations detected on d 45 of gestation. The concentration remains unchanged after d 45 during the remainder of the gestational period. These results are consistent with those resulting in a previous study indicating there is a peak concentration of PAG at 48.6 ± 5.0 d of gestation in does (Gonzalez et al., 2000). Similarly, there is a peak concentration of plasma PAG in does at 8 weeks of gestation and after parturition, the concentration decreases to the basal values within 2 weeks of lactation (Tandiya et al., 2013). Results from the present study are also consistent with those from earlier studies where there was utilization of PAG antisera in a radioimmunoassay (RIA) system for determination of pregnant and non-pregnant does on about d 21 after breeding (Batalha et al., 2001a; Barbato et al., 2009). As determined using a heterologous double-

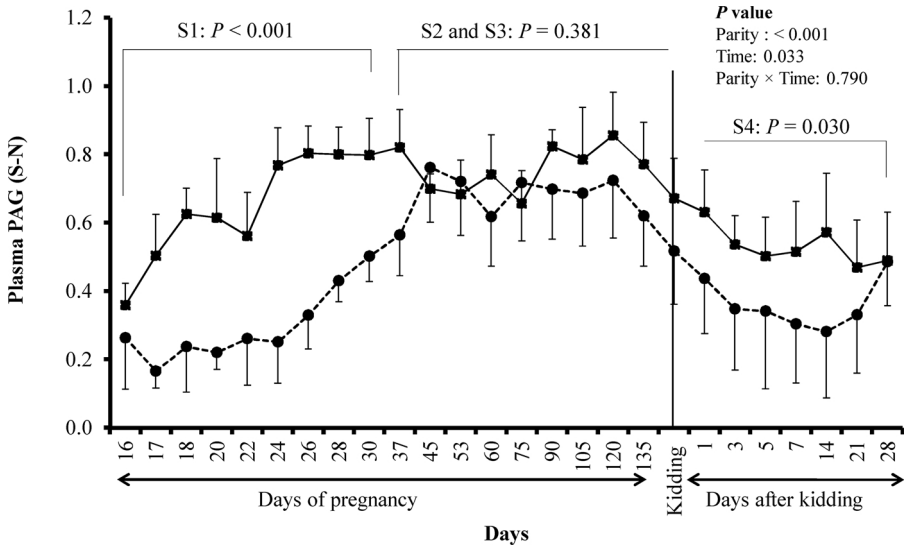

Fig. 5. Profiles of circulating caprine pregnancy-associated glycoprotein (caPAG) (mean signal-noise; S-N ± SEM) quantified using an ELISA for does with a single foetus that were nulliparous (dotted line and closed circle; $n = 7$) or multiparous (solid line and closed square; $n = 8$) Barbari does during and after gestation; S1 = pregnancy stage 1 (early pregnancy: to d 37); S2 and S3 = pregnancy stage 2 (mid-pregnancy: from d 45 to d 90) and pregnancy stage 3 (late-pregnancy: from d 105 to day of parturition), respectively; S4 = days after parturition (d 1 to 28 postpartum); Type of parity represents nulliparous or multiparous Barbari does.

antibody RIA utilising rabbit antiserum raised against a mixture of caprine 55 and 59 kDa PAG subunits as the first antibody, Karen et al. (2003) reported that d 22 post-mating was the first gestational day for detection of PAGs in ewes.

The binucleate cells of placenta, the major source of maternal PAG, are first observed at d 18 of pregnancy in goats and the proportion of these cells increases rapidly from less than 1% to 16% by d 19, and 22% by d 23 of pregnancy (Wango et al., 1990). Based on this observation, it is expected that a detectable amount of PGA in peripheral blood should be present around d 20 of pregnancy. Thus, the plasma profile of caPAG is consistent with the physiological changes related to the population of placental binucleate cells during early pregnancy in goats.

There are numerous factors that may affect the concentration of PAG throughout gestation in goats and other farm animals. In the present study, there was a markedly greater concentration of caPAG in multiparous does with two foetuses compared to does with a single foetus. This difference was first observed at d 45 of pregnancy (1.74 fold; S-N = 1.22 ± 0.21 compared with 0.70 ± 0.04) and the pattern of greater concentrations was sustained until the end of gestation. Results from the present study where there was a greater caPAG concentration in does with two foetuses than one foetus are consistent with results from earlier studies with other farm animals such as cattle (Szelényi et al., 2015) and sheep (Ledezma-Torres et al., 2006). Szelényi et al. (2015) reported that there was a 1.36 fold greater concentration of PAG in cows with two foetuses compared with a single foetus at 29 to 42 d of gestation. Similarly, there was a greater plasma PAG concentration in ewes with two foetuses than those with a single foetus from about 8 weeks of gestation until 1 week after parturition (Ledezma-Torres et al., 2006). In contrast to these results, results of other studies indicate there is an effect of litter size on plasma PAG concentrations either only during 15 to 19 week of gestation in goats (de Sousa et al., 1999), at week 21 of pregnancy in ewes (Ranilla et al., 1997) or from 4 weeks before until 4 weeks after parturition in cattle (Roberts et al., 2017). It is suggested that the greater PAG concentrations in dams with two foetuses are the result of a larger utero-placental mass and increased numbers of binucleate cells (Echternkamp et al., 2006). According to Hayden et al. (1979), the total weight of placentomes increases with total foetal weight and hence with the number of foetuses. Thus, the increase in placental mass and number of binucleate cells with litter size explain why there are greater concentrations of caPAG in does with multiple foetuses compared to those with a single foetus.

In the present study, there was a greater plasma caPAG concentration in multiparous compared with nulliparous does during early gestation. These results are consistent with results from an earlier study where there was a trend for greater PAG concentrations in pregnant cows than heifers on d 24 of pregnancy (Reese et al., 2018). Inconsistent with these findings, there were no differences detected in PAG concentrations in multiparous and nulliparous does when there was use of a homologous or heterologous RIA system for quantification of PAG concentrations until d 133 of gestation (Batalha et al., 2001a). In the studies with cattle, variable results of the effect of parity on plasma PAG concentration were observed. This includes, lesser PAG concentrations in multiparous compared with nulliparous cows (Ricci et al., 2015; Pohler et al., 2016) or absence of parity effect on PAG concentrations (Batalha et al., 2001a; Serrano et al., 2009). The variation in the results may be due to the difference in the target species and the methodologies (including antisera) used for PAG quantifications. The effect of maternal reproductive status (nulliparous or multiparous) on plasma caPAG concentration in goats during early pregnancy and after parturition needs further investigation.

According to Wooding (1992), there is no difference in placental binucleate cell population between multiparous and nulliparous animals. The average placental weight, cotyledon surface area and cotyledon efficiency, however, are less in first-parity than

multiparous does (Uğur and Önder, 2016). Such anatomical differences may result into increased production of caPAG in multiparous compared to nulliparous does.

Pregnancy diagnosis through the determination of plasma PAG concentration in ruminants is sometimes complicated by the presence of PAGs in circulation for an extended period after parturition. This is mainly because of the variable degree of glycosylation of different PAG molecules (Klisch et al., 2005). Thus, when using plasma PAG for early pregnancy diagnosis, one must take into account the half-life and sustained concentrations of PAGs from the previous pregnancy during postpartum period.

With the ELISA system developed in the present study, there was a rapid postpartum decrease in circulating caPAG concentrations so that its level reaches the concentrations quantified were basal within 3 weeks after parturition. This information coupled with the fact that it takes more than 45 days to reinitiate oestrous cycles and for involution of the postpartum uterus to the pre-gravid stage after parturition in does (Goel et al., 2016), it appears that as observed in cattle (de Sousa et al., 2003), caPAGs residual concentrations of caPAG from the previous gestation are not a problem for early pregnancy diagnosis in goats with the ELISA system developed in the present study. These results verify use of the caPAG assay that can be a reliable option for pregnancy determination in goats.

5. Conclusions

The results using the ELISA system developed in the present study indicate this is a useful technique for early pregnancy detection around d 22 of gestation and for determination of does with two or one foetuses at ≥ d 45 of gestation in multiparous Barbari goats. The maternal caPAG concentrations increased in circulation during early gestation with the greatest concentrations at d 45 of gestation and concentrations were sustained until the time of parturition. Maternal reproductive status (parity) affected the gestational profile of caPAG concentrations in peripheral blood with a greater concentration of PAG in multiparous compared to the nulliparous does. Due to the rapid disappearance of circulating caPAG following parturition, residual caPAG from the previous pregnancy should not be a problem with detection of pregnancies that occur as a result of a subsequent breeding. Further studies are required to evaluate the application of caPAG assay as a routine pregnancy diagnosis method in commercial goat flocks.

Declaration of competing interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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